

Conformation of β -Casein B

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Molecular weight and viscosity studies of β -casein B in 0.14 M NaCl, 0.02 M EDTA, pH 7, 25°, and in 6.0 M Guanidine HCl, pH 7, 25°, have led to the conclusion that the protein has a random coil conformation in both of these solvents.

Bovine β -casein, one of the major components of whole casein, has interesting physical properties in solution. The optical rotation studies of Kresheck (1) and Herskovits (2) indicate that the protein is loosely folded even in the absence of a denaturing agent. In dilute neutral salt solution β -casein is a monomer at 4°, but it is highly aggregated at room temperature, as was shown by Sullivan *et al.* (3) and Payens and Van Markwijk (4). In 1963 Aschaffenburg reported that β -casein has three genetic variants, named A, B, and C, which were detected by mobility differences upon starch gel electrophoresis in an alkaline buffer (5). More recently, Peterson and Kopfler have shown that "genetic variant A," if obtained from pooled milk, can be resolved into three bands by polyacrylamide gel electrophoresis in an acidic buffer (6). Genetic variants B and C, however, migrated as single bands in both acidic and alkaline buffers, indicating that, as far as can be determined by electrophoresis, they are single genetic variants. It seems likely that much of the earlier work on β -casein was done on mixtures of variants. Since the genetic variants of bovine α_{s1} -casein and β -lactoglobulin differ markedly in some of their properties (e.g., aggregation behavior) in solution (7, 8), it was considered desirable to repeat the earlier molecular weight determinations on a sample of β -casein B obtained from a single cow.

In this study the previous conformation studies were extended by viscosity measurements since they suggested that β -casein might be a random coil, and the results of the recent viscometric study by Tanford *et al.* (9) of proteins in 6 M guanidine hydrochloride strongly suggest that this is a possible conformation for proteins. Comparison of the value of the intrinsic viscosity of a protein with that expected from the intrinsic viscosity-molecular weight relationship found by Tanford *et al.* allows determination of the conditions under which the protein has a conformation approximating a random coil.

MATERIALS AND METHODS

β -Casein B was prepared from the milk of an individual cow, homozygous for that variant, by the method of Thompson (10). Polyacrylamide gel electrophoresis (10) revealed the presence of only traces of contaminants. Guanidine hydrochloride (Gu·HCl) was prepared by acidification of the carbonate (Eastman Organic Chemicals²). All other reagents were of analytical grade or the equivalent. The β -casein concentration, used in calculating the reduced viscosity in dilute salt, was determined by dry weight measurement at 107–109°. The protein concentration in 6 M Gu·HCl pH 7, was determined spectrophotometrically by using the value of the absorptivity of 4.4 dl/gm-cm at 278 m μ ; this value was determined by measurement of the absorbance of a solution of known

¹ Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

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concentration (in dilute salt) after dilution with concentrated $\text{Gu}\cdot\text{HCl}$ to 6 M $\text{Gu}\cdot\text{HCl}$.

Molecular weights were determined by the meniscus depletion sedimentation equilibrium technique of Yphantis (11). The experiments were performed in a Spinco model E ultracentrifuge equipped with interference optics; aluminum-filled Epon double-sector centerpieces, 12 mm thick, were routinely used. Fringe displacements were measured as a function of radial distance, r , by use of a Gaertner microcomparator. The molecular weight was obtained by use of the following relationship (11):

$$\frac{d \ln f}{d(r^2)} = \frac{M_w(1 - \bar{v}_p)}{1 + c \left(\frac{\partial \ln y}{\partial c} \right)_{P,T}} \cdot \frac{\omega^2}{2RT}, \quad (1)$$

where f is the fringe displacement, M_w is the weight-average molecular weight at the point, r , \bar{v} is the partial specific volume of the solute, ρ is the solution density, ω is the angular velocity, R is the gas constant in cgs units, T is the absolute temperature, P is the pressure, and y is the activity coefficient of solute based on the same scale as c , the solute concentration. If r does not change very much,

$$d(r^2) \cong 2 \bar{r} dr,$$

where \bar{r} is the average value of r for the range involved (11). Then the slope of a plot in $\ln f$ vs. r is directly proportional to molecular weight. The error arising from use of Eq. (2) was about 0.3% in these studies. The data obtained were of sufficient quality that a line could be drawn by eye that represented all the points. Use of the slope resulted in a weight-average molecular weight for the average concentration between the first and last points. This concentration was about 0.5 gm/liter for the denaturing solvents used, and 1.2 g/liter for silute salt solution. The nonideality term, $c[(\partial \ln y)/(\partial c)]_{P,T}$, is small at such concentrations, but its neglect might have resulted in molecular weights in aqueous guanidine hydrochloride and urea about 5% too low (12); no claim is made for better accuracy than 5%.

Densities used in the molecular weight calculations were measured with a commercially obtained Robertson specific gravity bottle of about 20-ml capacity.

Viscosity measurements were made at $2.5 \pm 0.01^\circ$ and $25 \pm 0.01^\circ$ with Ostwald-Fenske viscometers having flow times for water of 100–300 seconds at 25° .

RESULTS AND DISCUSSION

Molecular weight. Figure 1 shows a plot of $\log f$ vs. r for β -casein B, in 0.14 M NaCl, 0.02 M EDTA, pH 7, 2.5° . The linearity of

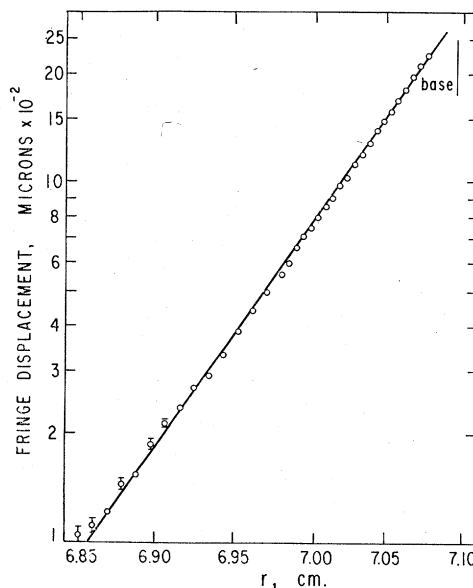


FIG. 1. Fringe displacement (logarithmic scale), in microns, at equilibrium, vs. r for β -casein B in 0.14 M NaCl, 0.02 M EDTA (pH 7), 2.5° . The error bars correspond to an estimated uncertainty of $\pm 5\mu$ in the determination of the fringe displacement.

the plot is indicative of homogeneity of the sample. A molecular weight of 23,400 is obtained from the slope of the plot when the value 0.733 ml/gm is used for the partial specific volume [obtained by correcting the \bar{v}_2 of 0.741 ml/gm at 25° (13) for temperature dependence using $d\bar{v}_2/dT = 0.000365$ ml/gm deg (14)]. Values of $M_w(1 - \bar{v}_2\rho)$ for β -casein B in denaturing media are given in Table I. Molecular weights very close to 24,000 are obtained if the value 0.741 ml/gm is used for \bar{v}_2 (the value at 25° uncorrected for preferential solvation) indicating that the effect of preferential solvation (15) on \bar{v}_2 is small or negligible for β -casein B in concentrated $\text{Gu}\cdot\text{HCl}$ or urea. The molecular weight of 23,000–24,000 found in this study is in excellent agreement with the value of 24,000 obtained by sedimentation diffusion (3), the Archibald method (4), and by chemical methods (16). Since no attention was paid to genetic polymorphism in the previous physical studies, it is likely that the good agreement indicates that all the genetic variants of β -casein are monomers at 2.5° in dilute neutral salt solution.

TABLE I
MOLECULAR WEIGHT RESULTS

	$M_w(1 - \bar{v}_2\rho) \times 10^{-3}$	$\rho(\text{gm/ml})$	$M_w \times 10^{-3}$
3 M Gu·HCl ^a	4.76	1.078	23.7 ^b
5 M Gu·HCl ^a	3.94	1.125	23.7 ^b
6.67 M Urea ^a	4.70, 4.35	1.102	25.6, 23.7 ^b
0.14 M NaCl, 0.02 M EDTA, pH 7, 2.5°	6.12	1.008	23.4 ^c

^a The solvent also contained 0.023 M sodium phosphate, pH 7; the temperature was 25°.

^b Calculated by using the value 0.741 ml/gm for \bar{v}_2 (13).

^c Calculated by using the value 0.733 ml/gm obtained by correcting the value 0.741 ml/gm for temperature dependence.

The agreement of the molecular weight obtained in this study with the generally accepted value is important because it has been our experience that β -casein is not stable in dilute neutral salt solution at 15° and above. A variety of methods were used to try to stabilize the protein, but none were completely successful. Degradation could be detected by the appearance of new bands on alkaline polyacrylamide gel electrophoresis that moved slower than the original band. It was found, however, that samples could be stored at 2.5° for 3 days without degrading; at 25° extensive degradation took place. Other evidence revealed degradation after 24 hours at 25°. Since the sedimentation equilibrium runs lasted about 3 days, it can be concluded from our results that β -casein B is quite stable at 2.5°.

Intrinsic viscosity. The viscosity data were fitted by the method of least squares to the expression:

$$\eta_{sp}/c = [\eta] + k[\eta]^2c, \quad (3)$$

where η_{sp}/c is the reduced viscosity in ml/gm, $[\eta]$ is the intrinsic viscosity in ml/gm, c is the protein concentration in gm/ml, and k is a dimensionless constant known as the Huggins constant. The viscosity results are illustrated in Fig. 2, and the values of $[\eta]$ and k obtained in this study are listed in Table II, along with the viscosity results for monomeric β -casein obtained by Sullivan *et al.* (3) and Payens and Van Markwijk (4) for comparison. Table II shows that our results at ionic strengths of 0.1 and 0.2 agree rather well with those obtained at $I = 0.2$ by Payens and Van Markwijk, but

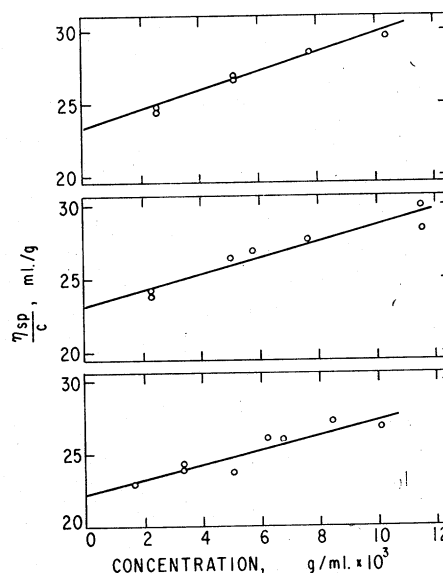


FIG. 2. Viscosity data for β -casein B in the following solvents: Upper: 0.04 M NaCl, 0.02 M EDTA (pH 7), 2.5°. Middle: 0.14 M NaCl, 0.02 M EDTA (pH 7), 2.5°. Lower: 6 M Gu·HCl, 0.1 M potassium phosphate (pH 7), 2.5°.

disagree with those at $I = 0.1$ obtained by Sullivan *et al.* The lack of agreement could stem from either a difference in the genetic variants studied or a difference in the preparative methods used.

The intrinsic viscosity of β -casein B in 6 M Gu·HCl, 0.1 M potassium phosphate, pH 7, 25°, has the value 22.2 ± 1.0 ml/gm (Table II). Tanford *et al.* (9) have found recently that for a number of proteins in 6 M Gu·HCl (and mercaptoethanol) at 25°, the intrinsic viscosity is a function of n , the number of amino acid residues, and is

TABLE II
VISCOSITY RESULTS

Solvent	$[\eta]$, ml/gm	k
0.04 M NaCl, 0.02 M EDTA, pH 7, 2.5°	23.3	1.2
0.14 M NaCl, 0.02 M EDTA, pH 7, 2.5°	23.1	1.0
6 M Gu·HCl, 0.1 M potassium phosphate, pH 7, 25°	22.2 ± 1.0^a	1.0 ± 0.3^a
0.08 M NaCl, Veronal, $I = 0.1$, pH 7.78, 8°	14.9 ^b	—
NaCl, Veronal, $I = 0.2$, pH 7.5, 4°	23 ^c	0.93

^a Most probable error; the data for 6 M Gu·HCl showed the most scatter about the least squares line.

^b From Sullivan *et al.* (3).

^c From Payens and Van Markwijk (4).

given by the relation:

$$[\eta] = 0.716 n^{0.66}. \quad (4)$$

The value of the exponent, 0.66, is within the range 0.5–0.8 predicted by theory, and found experimentally, for random coils. This constitutes good evidence that proteins in 6 M Gu·HCl are, to a first approximation, random coils. It was recognized, however, that the results did not imply complete freedom of rotation about the bonds between the α -carbon and the neighboring amide groups. The intrinsic viscosity for β -casein B predicted by use of Eq. (4) is 24 ml/gm ($n = 202$; mol. wt. = 24,000; mean residue wt. = 119), a value quite close to the value 22 ml/gm actually found. Thus, β -casein B in 6 M Gu·HCl is quite well approximated by a random coil.

The intrinsic viscosity of β -casein B in 0.04 M NaCl (and 0.14 M NaCl), 0.02 M EDTA, 2.5°, is remarkably close to that found in 6 M Gu·HCl, suggesting that even in a benign solvent, the protein has a highly disorganized structure. It should be noted that β -casein is isolated from whole casein by chromatography on DEAE-cellulose with 3.3 M urea present to reduce interactions between α_{s1} -, β -, and κ -casein. This treatment apparently has little effect on the backbone organization of the casein since Herskovits (2) found that the optical rotation parameters of whole casein, which was prepared by precipitation in the isoelectric range, in the absence of urea, are approximately equal to the averaged values of the isolated components.

At pH 7, β -casein has a net negative

charge of 11 per molecule (13). Flexible charged molecules are sometimes extended at low ionic strengths because of repulsion of like charges, and the intrinsic viscosity would accordingly be a function of ionic strength (17, p. 489). Our results indicate that if a charge effect does influence the intrinsic viscosity of β -casein B, it is unimportant at the ionic strength 0.1 since a further increase in ionic strength to 0.2 causes no change in the intrinsic viscosity. An attempt was made to demonstrate the charge effect at very low ionic strength, but it failed because the protein is not a good buffer at pH 7, and the pH drop resulting from CO₂ absorption resulted in aggregation.

The value of the Huggins constant, k , for β -casein B is 1–1.2 in dilute neutral salt as well as in concentrated Gu·HCl. The value is in good agreement with the value 0.93 calculated from the results of Payens and Van Markwijk (4). It is slightly higher than the highest values (0.59–0.95) found by Tanford *et al.* (9) for proteins in 6 M Gu·HCl. The value of 0.35 has been found for a number of flexible polymers in good solvents, while the value 2 is characteristic of compact globular molecules (17, p. 392).

The intrinsic viscosity is a function of solvent-solute interactions as well as the inherent size and shape of a polymer. Therefore, to compare β -casein B in dilute salt with the protein in 6 M Gu·HCl, it is necessary to determine the dimensions the molecule would have if there were no interaction with solvent. The "unperturbed" dimensions can be estimated by use of the

following equation (18):

$$[\eta] = \Phi \langle r^2 \rangle_0^{3/2} M^{-1} \alpha^3, \quad (5)$$

where Φ is a universal constant for random coils and has a value of approximately 0.21 if the intrinsic viscosity has the units ml/gm, $\langle r^2 \rangle_0$ is the square of the unperturbed end-to-end distance in Å units; and α is a factor that corrects for thermodynamic nonideality. If A_2 , the second osmotic virial coefficient, is known, α is given by (18):

$$\frac{100 A_2 M}{[\eta]} = 188 \ln [1 + 0.866 (\alpha^2 - 1)]. \quad (6)$$

The minimum value for A_2 is usually zero, and the minimum value for α according to Eq. (6) would be about 1. Values of A_2 less than zero usually imply aggregation and incipient precipitation (19). If the value of 1 is used for α , our results indicate an upper limit for $\langle r^2 \rangle_0^{1/2}$ of about 137 Å in dilute salt. Tanford *et al.* made nonideality corrections for proteins in 6 M Gu·HCl by methods that did not require knowledge of A_2 (9). From their results, α in this solvent would be 1.1–1.2, and $\langle r^2 \rangle_0^{1/2}$ for β -casein in 6 M Gu·HCl would be about 115 Å. Explanations can be offered for the difference between the values of $\langle r^2 \rangle_0^{1/2}$ but because of the lack of definite values for α in the solvents, they would not be justified.

The unusual conformation of β -casein B must be a result of its atypical amino acid composition; it has no disulfide bonds and one residue in six is proline (16). Ho and Chen (20) recently made viscosity measurements on α_{s1} -casein B, the most common genetic variant of the major component of whole casein. This protein also lacks disulfide bonds, but has only half the proline content of β -casein B. They found that the intrinsic viscosity of the monomer in 0.01 M KCl, pH 7, was 10–12 ml/gm, depending on the temperature. In contrast to β -casein B, the value increased to 19.2 ml/gm in 6 M Gu·HCl, 0.1 M KCl, pH 7.1, 25°. Thus α_{s1} -casein B, while not being a compact globular protein (i.e., $[\eta] = 3\text{--}4$ ml/gm), is more tightly folded than a random coil.

An interesting comparison can be made between the caseins, γ -globulin, and myosin.

The caseins, characterized by a high proline content, are readily cleaved by proteolytic enzymes (21), but γ -globulin is very resistant except for a small sensitive region of the heavy chain; myosin also has a small region that is particularly sensitive to enzymes. It is noteworthy that recent amino acid sequence studies on γ -globulin (22) and amino acid analyses of proteolysis products of myosin (23) have revealed in each case an unusually large amount of proline in the enzyme-sensitive region. It appears that proline residues must prevent close packing of the nearby residues, and thus render them readily available for enzymic attack.

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